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### (54) Process for the preparation of microspheres containing biologically active components

Verfahren zur Zubereitung von biologisch aktiven Komponenten enthaltenden Mikrosphären

Procédé pour la préparation de microsphères contenant des composés actifs biologiquement

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EP-A- 0 290 891 EP-A- 0 330 180  
EP-A- 0 412 554

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• JOURNAL OF CONTROLLED RELEASE, vol. 13,  
1990, pages 33-41; L.M. BENEDETTI et al.:  
"Microspheres of hyaluronic acid esters-  
fabrication methods and in vitro hydrocortisone  
release"  
• IDEM

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EP 0 517 565 B1

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## Description

[0001] An object of the invention is a process for the preparation of microspheres starting with biodegradable and bioabsorbable semisynthetic polymers and single molecules or mixtures of the same having pharmacological activity, allowing the chemical characteristics of the semisynthetic polymer used and the biological or pharmacological activity of the molecules to remain unaltered, guaranteeing controlled release depending on the hydrophilic properties of the chemical substituents present on the semisynthetic polymer.

## DESCRIPTION OF RELATED ART

[0002] Proteins and peptides are today considered to be an important base for therapeutic agents. Recombinant DNA technology has made it possible to produce many such macromolecular agents with interesting and useful biological and pharmacological properties. The enormous technological advancement in chemical synthesis has made many polypeptides with high pharmacological activity accessible. For these drugs however problems still persist regarding stability and their administration to man. When an active principle is administered to man, it is essential to guarantee that its pharmacological activity will remain unaltered and that its release will be controlled, in order to avoid undesirable side-effects. It is known that these macromolecular agents are not usually efficacious after oral administration, since they are rapidly degraded and deactivated by the proteolytic enzymes present in the gastrointestinal tract.

[0003] Even when these macromolecules do resist enzymatic digestion, their absorption is often very slight because of their large size. Other routes of administration, such as by nose, mouth, vagina, rectum and through the skin, have been used for the absorption of proteins and peptides but bioavailability proved to be low and variable on account of the intrinsic characteristics of the active principle. Consequently, these molecules are normally administered by the parenteral route, even though this route, too, has its disadvantages, which are mainly linked to rapid elimination from the bloodstream. Over the past decade remarkable progress has been made in pharmaceutical technology dedicated to the preparation of formulations which allow, on the one hand, for the intrinsic activity of proteins to be preserved and, on the other, for their controlled release (Langer R., Science 1527, 1990).

[0004] The use of synthetic or partially natural polymeric matrixes means that drug release is now reproducible, guaranteeing constant concentrations in the bloodstream, thereby avoiding repeated administrations with the consequent risk of side effects.

[0005] However, the use of these polymeric matrices has given rise to a series of new problems linked essentially to the very nature of the polymers used, such as their toxicity and that the toxicity of their degradation products, biocompatibility, the removal of deposits of undegradable debris.

[0006] Current research is aimed at identifying and developing bioabsorbable and biodegradable polymers, partly or wholly made of natural substances, capable of the controlled release of biologically and pharmacologically active molecules, which are able to protect these molecules from degradation while allowing for their prolonged release, and without affinity for fibrous organic tissues which might alter their release properties, they should not present undesirable reactivity towards pharmacologically active molecules, and neither they, nor their degradation products should be immunogenic.

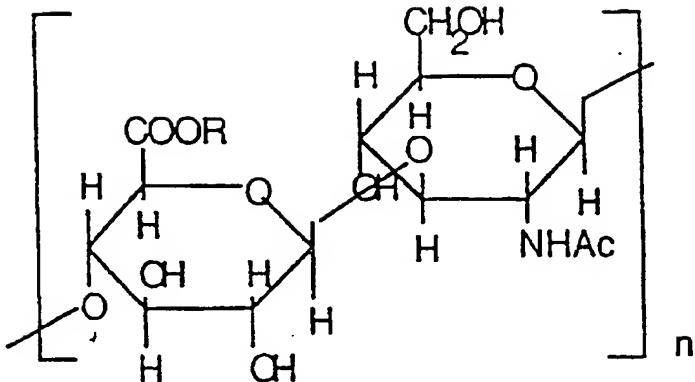
[0007] Examples of natural polymers widely used as release systems for pharmacologically active molecules such as hyaluronic acid have been described in US Patent 4,851,521 and US Patent 4,965,353; alginic acid in EP Publ. No. 0251905; chitosan in EP Publ. No. 0341745; and gellan of the acidic polysaccharides.

[0008] Hyaluronic acid is a polysaccharide widely distributed in animal organisms, and is constituted by alternating units of D-glucuronic acid and N-acetyl-D-glucosamine. The mean molecular weight varies between 2 x 10<sup>4</sup> and 7 x 10<sup>6</sup> according to the purification method used. Hyaluronic acid has been used, as described, for example, in US patent 4,772,419, to prevent adhesion and tissue enlargement. US patent 4,636,524 also describes a release system for biologically active substances to be dispersed in the molecular "cage" formed by the meshwork of hyaluronic acid gel. Hyaluronic acid has also been described in the literature as a carrier for drugs trapped in the biodegradable, collagen-based matrix. In U.S. patents Nos. 4,851,521 and 4,965,353, a chemical method is reported for the esterification of carboxy groups of hyaluronic acid with therapeutically active or inactive alcohols (HYAFF). With this chemical modification, the chemico-physical properties of the polymer change too, such as the hydrophobic and hydrophilic properties of the polymer, while its chemical structure of the polysaccharide remains unaltered, as set forth below.

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### Hyaluronic Acid Esters

[0009] Moreover, various methods for the preparation of systems for the controlled release of biologically active substances are described in the literature.

[0010] For instance microspheres containing biologically and/or pharmacologically active molecules are described in the PCT patent WO 89/03207. These release systems include the association of pharmacologically and/or biologically active molecules, denaturable like polypeptides, with natural polymers (starch, gelatin or albumin). The use of hyaluronic acid and/or its derivatives as a polymeric carrier to prepare formulations (microspheres) to be used for the release of active substances through the mucosa, such as the vaginal or nasal mucosa, is not described.

[0011] The in vitro characterization of the release of pharmacologically active substances, not of a proteic or glycosphingolipid nature, from microspheres prepared with hyaluronic acid esters are described in the paper "Microspheres of Hyaluronic Acid Esters Fabrication Methods and in vivo Hydrocortisone Release" by Benedetti et al., Journal of Controlled Release 13, 33-41 (1990).

[0012] Of the various technologies developed for the manufacture of microspheres, the most successful have been the "evaporation" and "extraction" techniques. Both of these processes require the preparation of an emulsion of two unmixable liquids. The emulsifying phase, known as the discontinuous phase, is constituted by microdroplets of a solvent containing modified hyaluronic acid and the substance, or suitable mixtures of biologically and/or pharmacologically active substances. The other phase of the emulsion, known as the continuous phase, is represented by a second solvent in which the microdroplets are homogeneously dispersed. When the emulsion is stable, the discontinuous phase is removed either by evaporation or extraction according to the type of technique employed. It is possible to obtain release systems with different characteristics according to how the biologically active substance or mixture of substances are incorporated in the microspheres. For example, when the active principle is physically dispersed in the polymer matrix constituting the microspheres, its release is controlled by the diffusion rate of the biologically and/or pharmacologically active substance through the polymer network.

[0013] EP-A-0330180 discloses a controlled release polylactic type preparation, notably microspheres. The process disclosed is a solvent evaporation process.

[0014] The paper by Benedetti et al. (1990) refers, in particular, to the possibility of obtaining microspheres by evaporation, because the extraction method produces microspheres with very porous surfaces; and, consequently, the polymeric matrix of which they are constituted has no control over the release of the active principle (corticosteroid). The present invention, surprisingly, offers the possibility of producing, by extraction, smooth-surfaced microspheres which therefore have more control over the release of the substances which are incorporated therein.

[0015] Another advantage of the extraction technique of the present invention lies in the possibility of obtaining microspheres with a notably smaller diameter than those cited in Benedetti et al.

[0016] It therefore follows that the use of said microspheres, unlike those with a larger diameter, guarantees a greater total surface area and therefore more contact with the tissues to be treated.

[0017] The present invention describes the preparation of microspheres containing molecules of a proteic nature (such as calcitonin, insulin, immunoglobulin, trophic factors such as hCNTF/hNGF) and/or of a glycosphingolipid nature

(natural gangliosides or their chemical derivatives), that is, the preparation of compounds which are quite different in structure, chemical-physical characteristics and stability from the compound (corticosteroid) discussed in the paper by Benedetti et al. It has been demonstrated, surprisingly, that by using this release system, the proteins associated with the polymer do not undergo degradation and maintain their biological activity.

5 [0018] The present invention also demonstrates the incorporation of high-molecular-weight molecules, i.e., the molecular weight of the incorporated molecules is considerably higher than that of the corticosteroid.

[0019] The possibility of preparing microspheres from HYAFF derivatives with distinct chemical-physical (hydrophilic/ hydrophobic) characteristics, chosen according to the chemical-physical characteristics of the biologically active molecule used, and where the biologically active molecule is to be applied, the release time and consequent 10 action of the pharmacologically active molecule, is described according to the present invention. As a result of the present invention, it is possible to prepare microspheres from suitable mixtures of HYAFF polymer and pharmacologically and biologically active substances, specifically designed according to the type of administration, the type of active substance and the desired action of time. It is also possible to prepare microspheres where the pharmacologically and/or biologically active substance or mixture of substances is superficially adsorbed. The principles described above 15 for microspheres and the possibility of a pharmacological interaction aimed at the site of action are valid in this case too.

#### SUMMARY OF THE INVENTION

20 [0020] The microspheres prepared according to claim 1 exercise a biological and/or pharmacological action, and can be prepared according to the desired site of their desired time of release, the type of biologically and/or pharmacologically active agent to be released, while leaving intact, the biological and pharmacological properties of the agents.

[0021] The invention thus provides a solvent extraction process for producing microspheres for the controlled 25 release of a biologically active molecule, which comprise a biologically active molecule and an ester of hyaluronic acid or mixtures of said esters of hyaluronic acid, wherein said biologically active molecule is surrounded by or adhered to said ester of hyaluronic acid or mixtures thereof, and wherein said microsphere has a diameter of between 1  $\mu\text{m}$  to 100  $\mu\text{m}$  and shows a smooth surface, which comprises

30 dissolving an ester of hyaluronic acid or mixtures of said esters of hyaluronic acids and said biologically active molecule in an aprotic solvent to form a solution;

adding said mixture to high viscosity mineral oil containing a nonionic surfactant to produce a continuous phase;

35 adding ethyl acetate to said continuous phase to extract said aprotic solvent thereby producing said microspheres.

[0022] Preferred embodiments correspond to claims 2-14.

#### 35 BRIEF DESCRIPTION OF THE DRAWINGS

[0023]

40 Figure 1 shows the levels of calcium in plasma (%) after vaginal administration of calcitonin in solution.

Figure 2 shows the levels of calcium in plasma (%) after vaginal administration of calcitonin associated with microspheres of HYAFF-11 and HYAFF-11 p75.

Figure 3 shows the effect of different doses of HYAFF-11 microspheres containing insulin on plasma glucose decrease after nasal administration in sheep.

45 Figure 4 shows the levels of insulin in plasma after nasal administration to sheep of different doses of HYAFF-11 microspheres.

Figure 5 shows the plasma levels of GM<sub>1</sub> after intramuscular administration to rabbits, on its own and with microspheres of different diameters.

Figure 6 shows the NGF released from microspheres of HYAFF-11 p75.

50 Figure 7 shows the NGF released from microspheres of HYAFF-11.

Figure 8 shows the NGF released from microspheres of HYAFF-7.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

55 [0024] The microspheres prepared according to the present invention have a particle size of between 1 and 100  $\mu\text{m}$ , preferably between 1 and 15  $\mu\text{m}$ , and they have smooth surfaces. The following examples are purely illustrative of how to obtain the microspheres according to the present invention and their use and shall not be construed as limiting the scope of the invention.

Example 1

[0025] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353) is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying between 5 and 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide such as human insulin, at a predetermined concentration, for example 5 I.U. per mg of polymer, is added to the solution. The mixture obtained will be referred to hereinafter as the discontinuous phase. At the same time, a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

10 [0026] This mixture will be referred to hereinafter as the continuous phase.

[0027] The continuous phase is kept at 25°C while being stirred at a fixed speed of 1000 RPM, then the discontinuous phase, prepared as previously described, is added to it. In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0028] After 15 minutes of stirring, acetylacetate is added.

15 [0029] This solvent mixes perfectly with the two phases of the emulsion but it is a nonsolvent for the polymer and the human insulin polypeptide. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction the stirring speed is set at 1400-1500 RPM for 10 minutes and then lowered to 500 RPM. The suspension thus obtained continues to be stirred while being pumped with a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is

20 pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0030] In these working conditions the resulting mean particle size is 10 µm.

[0031] The quantity of incorporated insulin is 4 IU per mg of microsphere.

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Example 2

[0032] A hyaluronic acid ester wherein all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide at a concentration varying from 5 to 10% weight/volume, generally 7% p/v. The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time, a mixture is prepared in a suitable reactor of highviscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% p/v.

[0033] This mixture will be referred to hereinafter as the continuous phase. The continuous phase is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

35 [0034] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

After stirring for 15 minutes, ethyl acetate is added.

[0035] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C. The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M), containing a concentration of insulin such that a protein titer of 2 U.I per mg of suspended microspheres is reached. After 15 minutes stirring with a semiautomatic system the suspension is immersed in liquid nitrogen until it is completely frozen.

[0036] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

50 [0037] The mean particle size is 15 µm. The quantity of incorporated insulin is 2 IU per mg di microsfere.

Example 3

[0038] A hyaluronic acid ester wherein 75% of the carboxy groups of hyaluronic acid are esterified with benzyl alcohol while the remaining part is salified with sodium (HYAFF-11 p75, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide such as human insulin, at the predetermined concentration, for example 5 I.U. per mg of polymer, is added to the solution. The mixture thus obtained will be referred to

hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0039] This mixture will be referred to hereinafter as the continuous phase. The continuous phase is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0040] After stirring for 15 minutes, ethyl acetate is added.

[0041] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the human insulin polypeptide. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0042] The mean particle size is 20 µm.

[0043] The quantity of incorporated insulin is 4 IU per mg of microspheres.

#### 20 Example 4

[0044] A hyaluronic acid ester wherein all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, such as human insulin, at the predetermined concentration, for example 5 I.U. per mg of polymer, is added to the solution. The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0045] This mixture will be referred to hereinafter as the continuous phase. The continuous phase is kept at temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0046] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0047] After stirring for 15 minutes, ethyl acetate is added.

[0048] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the human insulin polypeptide. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained is stirred while being pumped by a screw pump, through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0049] The dimensions of the microspheres and the mean particle size is 30 µm.

[0050] The quantity of incorporated insulin is 4 I.U. per mg of microspheres.

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#### Example 5

[0051] A hyaluronic acid ester wherein all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353) is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable container of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0052] This mixture will be referred to hereinafter as the continuous phase. It is kept at temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0053] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0054] After stirring for 15 minutes, ethyl acetate is added.

[0055] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer. It

has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once filtration is complete, it is pumped through a normal-hexane filter, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0056] The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M), containing a concentration of insulin such that a protein titer of 2 I.U. per mg of suspended microspheres is reached. After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension has completely frozen.

[0057] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

[0058] The mean particle size is 30  $\mu$ m.

[0059] The quantity of incorporated insulin is 2 I.U. per mg of microspheres.

15 Example 6

[0060] A hyaluronic acid ester wherein all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example Nerve Growth Factor (NGF), at the predetermined concentration, for example 0.01% of the weight of the polymer mass, is added to the solution. The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0061] This mixture will be referred to hereinafter as the continuous phase. It is kept at temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it. In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0062] After stirring for 15 minutes, ethyl acetate is added.

[0063] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer and the polypeptide NGF. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0064] In these working conditions the resulting mean particle size is 10  $\mu$ m.

[0065] The quantity of incorporated NGF is 10 ng per mg of microspheres.

40 Example 7

[0066] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example Nerve Growth Factor (NGF), at a predetermined concentration, for example 0.01% of the weight of the polymer mass, is added to the solution. The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0067] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0068] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0069] After stirring for 15 minutes, ethyl acetate is added.

[0070] This solvent can be mixed perfectly with the two emulsion phases but it is not a solvent for the polymer. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being

a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C. The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M), containing a concentration of NGF such that a protein titer equal to 0.01% in weight of the suspended microspheres is reached. After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension is completely frozen.

5 [0071] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.  
 [0072] The mean particle size is 10  $\mu\text{m}$ .  
 [0073] The quantity of incorporated NGF is 10 ng per mg of microspheres.

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Example 8

[0074] A hyaluronic acid ester where 75% of the carboxy groups of hyaluronic acid are esterified with benzyl alcohol while the remaining part is salfified with sodium (HYAFF-11 p75, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example NGF, at the predetermined concentration, for example 0.01% of the weight of the polymer mass, is added to the solution. The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

20 [0075] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0076] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0077] After stirring for 15 minutes, ethyl acetate is added.

25 [0078] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the NGF polypeptide. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any possible residues of surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0079] The mean particle size is 15  $\mu\text{m}$ .

30 [0080] The quantity of incorporated NGF is 10 ng per mg of microspheres.

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Example 9

[0081] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example NGF, at the predetermined concentration, for example 0.01% of the weight of the polymer mass, is added to the solution. The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

40 [0082] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0083] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0084] After stirring for 15 minutes, ethyl acetate is added.

45 [0085] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or for the NGF polypeptide. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0086] The mean particle size is 30  $\mu\text{m}$ .

[0087] The quantity of incorporated NGF is 10 ng per mg of microspheres.

Example 10

5 [0088] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

10 [0089] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it. In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0090] After stirring for 15 minutes, ethyl acetate is added.

15 [0091] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 for 10 minutes, then lowered to 500 RPM. The suspension thus obtained is stirred while being pumped by screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the

20 double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0092] The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M), containing a concentration of NGF such that a protein titer equal to 0.01% in weight of the suspended microspheres is reached. After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension is completely frozen.

[0093] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

[0094] The dimensions of the microspheres and the mean particle size is 30 µm.

[0095] The quantity of incorporated NGF is 10 ng per mg of microspheres.

30 Example 11

[0096] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a

35 polypeptide, for example CNTF (Ciliary Neuronotrophic Factor), at the predetermined concentration, for example 0.01% of the weight of the polymer mass, is added to the solution.

[0097] The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

40 [0098] This mixture will be referred to hereinafter as the continuous phase. It is kept at temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0099] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0100] After stirring for 15 minutes, ethyl acetate is added.

45 [0101] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the polypeptide CNTF. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter

50 of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0102] In these working conditions the resulting mean particle size is 10 µm.

[0103] The quantity of incorporated CNTF is 10 ng per mg of microspheres.

55 Example 12

[0104] A hyaluronic acid ester, where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol

(HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

5 [0105] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0106] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0107] After stirring for 15 minutes, ethyl acetate is added.

10 [0108] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

15 [0109] The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M), containing a concentration of CNTF (Ciliary Neuronotrophic Factor) such that a protein titer equal to 0.01% in weight of the suspended microspheres is reached. After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension is completely frozen.

20 [0110] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

[0111] The mean particle size is 10  $\mu\text{m}$ .

[0112] The quantity of incorporated CNTF is 10 ng per mg of microspheres.

25 Example 13

[0113] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example CNTF (Ciliary Neuronotrophic Factor), at the predetermined concentration, for example 0.01% of the weight of the polymer mass, is added to the solution.

[0114] This mixture will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

30 [0115] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0116] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0117] After stirring for 15 minutes, ethyl acetate is added.

40 [0118] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the polypeptide CNTF. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

45 [0119] The mean particle size is 30  $\mu\text{m}$ .

[0120] The quantity of incorporated CNTF is 10 ng per mg of microspheres.

50 Example 14

[0121] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

55 [0122] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and

stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0123] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0124] After stirring for 15 minutes, ethyl acetate is added.

5 [0125] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being 10 a solvent with the double action of "drying" the preparation and of solubilizing any possible residues of surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

10 [0126] The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M) containing a concentration of CNTF (Ciliary Neuronotrophic Factor) such that a protein titer equal to 0.01% in weight of the suspended microspheres is reached. After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension is completely frozen.

[0127] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

[0128] The mean particle size is 30 µm.

[0129] The quantity of incorporated CNTF is 10 ng per mg of microspheres.

20 Example 15

[0130] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a 25 polypeptide, for example NGF at the predetermined concentration, for example 0.01% of the weight of the polymer mass, and a ganglioside mixture having as major components, GM1 21%, GD12 40%, GD1b 16% and GT1b 19% (Cronassial®) are added at a ratio of 1:1000 in terms of weight.

[0131] The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel® R, a non-ionic surface-active agent, 30 at a concentration of 1% w/v.

[0132] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0133] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

35 [0134] After stirring for 15 minutes, ethyl acetate is added.

[0135] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the polypeptide NGF or the GA mixture (Cronassial). It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to 40 be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0136] In these working conditions, the dimensions of the microspheres and the mean particle size is 10 µm.

45 [0137] The quantity of incorporated NGF and GA is 10 ng and 10 µg respectively per milligram of microspheres.

Example 16

[0138] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a ganglioside mixture (Cronassial®) at the predetermined concentration, for example 20% of the weight of the polymer, is added to the solution. The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel® R, a non-ionic surface-active agent, at a concentration of 1% w/v.

55 [0139] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it. In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and

continuous) is about 1 to 16.

[0140] After stirring for 15 minutes, ethyl acetate is added.

[0141] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the ganglioside mixture. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0142] The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M), containing a concentration of NGF such that a protein titer equal to 0.02% in weight of the suspended microspheres is reached, and such that the weight ratio of 1:1000 (NGF:gangliosides) is maintained.

[0143] After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension is completely frozen. Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

[0144] The mean particle size is 10 µm.

[0145] The quantity of incorporated NGF and GA is 20 ng and 20 µg, respectively, per milligram of microspheres.

#### Example 17

[0146] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example NGF at the predetermined concentration, for example 0.01% of the weight of the polymer mass, and a ganglioside mixture (Cronassial®) are added to the solution at a weight ratio of 1:1000 (NGF:Cronassial®). The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel®, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0147] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0148] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0149] After stirring for 15 minutes, ethyl acetate is added.

[0150] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer, the polypeptide (NGF) or the GA mixture. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0151] The dimensions of the microspheres and the mean particle size is 30 µm.

[0152] The quantities of incorporated NGF and GA are 10 ng and 10 µg respectively per milligram of microspheres.

#### Example 18

[0153] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a ganglioside mixture (Cronassial®) at the predetermined concentration, for example 20% of the weight of the polymer, is added to the solution. The solution thus obtained will be referred to hereinafter as the discontinuous phase.

[0154] At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel®, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0155] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0156] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0157] After stirring for 15 minutes, ethyl acetate is added.

[0158] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the ganglioside mixture. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0159] The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M), containing a concentration of NGF such that a protein titer equal to 0.02% in weight of the suspended microspheres is reached and such that the weight ratio of 1:1000 (NGF:gangliosides) is maintained. After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension is completely frozen.

[0160] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

[0161] The mean particle size is 30 µm.

[0162] The quantities of incorporated NGF and GA are 20 ng and 20 µg respectively per milligram of microspheres.

#### Example 19

[0163] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with dodecyl alcohol (HYAFF-73, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, to the solution are added a polypeptide, for example NGF at the predetermined concentration, for example 0.01% of the weight of the polymer mass, and a ganglioside mixture (Cronassial®) in a weight ratio of 1:1000 (NGF:Cronassial®). The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel®, a non-ionic surface-active agent, at a concentration of 1% w/v. This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0164] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0165] After stirring for 15 minutes, ethyl acetate is added.

[0166] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the polypeptide (NGF) or the GA mixture (Cronassial®). It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C. In these working conditions the resulting mean particle size is 20 µm.

[0167] The quantities of incorporated NGF and GA are 10 ng and 10 µg respectively per milligram of microspheres.

#### Example 20

[0168] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, to the solution are added a polypeptide, for example NGF at the predetermined concentration, for example 0.01% of the weight of the polymer mass, and a monosialoganglioside GM1 in a weight ratio of 1:1000 (NGF:GM1).

[0169] The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel®, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0170] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0171] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0172] After stirring for 15 minutes, ethyl acetate is added.

[0173] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the polypeptide (NGF) or the monosialoganglioside GM1. It has been proven that the volume of extracting solvent

needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0174] In these working conditions the resulting mean particle size is 10 µm. The quantities of NGF and monosialoganglioside GM1 are 10 ng and 10 µg respectively per milligram of microspheres.

10 Example 21

[0175] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, to the solution are added a polypeptide, for example NGF at the predetermined concentration, for example 0.01% of the weight of the polymer mass, and monosialoganglioside GM1 in a ratio of 1:1000 (NGF:GM1). This mixture will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel®<sup>®</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0176] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0177] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0178] After stirring for 15 minutes, ethyl acetate is added.

[0179] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer, the polypeptide (NGF) or the monosialoganglioside GM1. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained is stirred while being pumped by screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0180] The mean particle size is 30 µm.

[0181] The quantities of NGF and monosialoganglioside GM1 are 10 ng and 10 µg respectively per milligram of microspheres.

35 Example 22

[0182] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example CNTF, at the predetermined concentration, for example 0.01% of the weight of the polymer mass, and a ganglioside mixture (Cronassial®) are added to the solution in a ratio of 1:1000 (CNTF:gangliosides). The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacel®<sup>®</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0183] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0184] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0185] After stirring for 15 minutes, ethyl acetate is added.

[0186] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the polypeptide CNTF. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0187] In these working conditions the resulting mean particle size is 10  $\mu\text{m}$ . The quantity of incorporated CNTF and GA is 10 ng and 10  $\mu\text{g}$  respectively per milligram of microspheres.

Example 23

5 [0188] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a ganglioside mixture at the predetermined concentration, for example 20% of the weight of the polymer, is added to the solution.

10 [0189] The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacet<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

15 [0190] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

20 [0191] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

25 [0192] After stirring for 15 minutes, ethyl acetate is added.

30 [0193] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the ganglioside mixture. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction the stirring rate was set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

35 [0194] The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength = 0.15M) containing a concentration of CNTF such that a protein titer equal to 0.02% of the weight of the suspended microspheres is reached, and such that the weight ratio of 1:1000 (CNTF:gangliosides) is maintained. After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension is completely frozen.

40 [0195] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

45 [0196] The mean particle size is 10  $\mu\text{m}$ .

50 [0197] The quantities of incorporated CNTF and GA are 20 ng and 20  $\mu\text{g}$  respectively per milligram of microspheres.

Example 24

40 [0198] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example CNTF at the predetermined concentration, for example 0.01% of the weight of the polymer mass, and a ganglioside mixture (Cronassial<sup>®</sup>) in a weight ratio of 1:1000 (CNTF:Cronassial<sup>®</sup>) are added to the solution. The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacet<sup>R</sup>, a non-ionic surface-active agent, at a concentration of 1% w/v.

45 [0199] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

50 [0200] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

55 [0201] After stirring for 15 minutes, ethyl acetate is added.

60 [0202] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer, the polypeptide (CNTF) or the GA mixture (Cronassial<sup>®</sup>). It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any possible residues of surfactant which may be present on the surface of the microspheres. The

product is then put in suitable containers and stored at 4°C.

[0203] The mean particle size is 30 µm.

[0204] The quantities of incorporated CNTF and GA are 10 ng and 10 µg respectively per milligram of micro-spheres.

5

Example 25

[0205] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with ethyl alcohol (HYAFF-7, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a ganglioside mixture (Cronassial®) at the predetermined concentration, for example 10% of the weight of the polymer, is added to the solution. The solution obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacet®, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0206] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0207] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0208] After stirring for 15 minutes, ethyl acetate is added.

[0209] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the ganglioside mixture. It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is passed through a normal-hexane filter, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the microspheres. The product is then put in suitable containers and stored at 4°C.

[0210] The microspheres thus prepared are suspended in a phosphate buffer solution (0.01M) (ionic strength 0.15 M), containing a concentration of CNTF such that a protein titer equal to 0.01% in weight of the suspended micro-spheres is reached and such that the weight ratio of 1:1000 (CNTF:gangliosides) is maintained. After 15 minutes of stirring with a semiautomatic system the container is immersed in liquid nitrogen until the suspension is completely frozen.

[0211] Once frozen, the suspension is freeze-dried for 24 hrs and the product stored at 4°C.

[0212] The mean particle size is 30 µm.

[0213] The quantities of incorporated CNTF and GA are 10 ng and 10 µg respectively per milligram of micro-spheres.

Example 26

[0214] A hyaluronic acid ester where all the carboxy groups of hyaluronic acid are esterified with benzyl alcohol (HYAFF-11, as described in the US patent No. 4,965,353), is dissolved in an aprotic solvent such as dimethylsulfoxide, at a concentration varying from 5 to 10% weight/volume, generally 7% w/v. Once the polymer has solubilized, a polypeptide, for example CNTF, at the predetermined concentration, for example 0.01% of the weight of the polymer mass, and the monosialoganglioside GM1 (Sygen) are added to the solution in a ratio of 1:1000 (CNTF:Sygen). The mixture thus obtained will be referred to hereinafter as the discontinuous phase. At the same time a mixture is prepared in a suitable reactor of high-viscosity mineral oil containing Arlacet®, a non-ionic surface-active agent, at a concentration of 1% w/v.

[0215] This mixture will be referred to hereinafter as the continuous phase. It is kept at a temperature of 25°C and stirred at a rate of 1000 RPM, while the discontinuous phase, prepared as previously described, is added to it.

[0216] In these conditions, emulsification of the two phases is instantaneous. The ratio between the two phases (discontinuous and continuous) is about 1 to 16.

[0217] After stirring for 15 minutes, ethyl acetate is added.

[0218] This solvent can be mixed perfectly with the two emulsion phases, but it is not a solvent for the polymer or the polypeptide CNTF or the monosialoganglioside GM1 (Sygen). It has been proven that the volume of extracting solvent needed to obtain complete extraction is two and a half times the total volume of the emulsion. To facilitate extraction, the stirring speed is set at 1400-1500 RPM for 10 minutes, then lowered to 500 RPM. The suspension thus obtained continues to be stirred, while being pumped by a screw pump through a filter press set at 1 atmosphere. Once this filtration is complete, it is pumped through a filter of normal-hexane, this being a solvent with the double action of "drying" the preparation and solubilizing any residue surfactant which may be present on the surface of the micro-